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Fission yeast *pak1*$^+$ encodes a protein kinase that interacts with Cdc42p and is involved in the control of cell polarity and mating

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A STE20/p65^{pak} homolog was isolated from fission yeast by PCR. The *pak1*$^+$ gene encodes a 72 kDa protein containing a putative p21-binding domain near its amino-terminus and a serine/threonine kinase domain near its carboxyl-terminus. The Pak1 protein autophosphorylates on serine residues and preferentially binds to activated Cdc42p both *in vitro* and *in vivo*. This binding is mediated through the p21 binding domain on Pak1p and the effector domain on Cdc42p. Overexpression of an inactive mutant form of *pak1* gives rise to cells with markedly abnormal shape with mislocalized actin staining. Pak1 overexpression does not, however, suppress lethality associated with cdc42-null cells or the morphologic defect caused by overexpression of mutant cdc42 alleles. Gene disruption of *pak1*$^+$ establishes that, like *cdc42*$^+$, *pak1*$^+$ function is required for cell viability. In budding yeast, *pak1*$^+$ expression restores mating function to *STE20*-null cells and, in fission yeast, overexpression of an inactive form of Pak inhibits mating. These results indicate that the Pak1 protein is likely to be an effector for Cdc42p or a related GTPase, and suggest that Pak1p is involved in the maintenance of cell polarity and in mating.

Keywords: cell morphology/mating pathway/protein kinase cascade/*Schizosaccharomyces pombe*/serine-threonine phosphorylation

Introduction

How a cell controls its shape is a fundamental question in biology. Many cells grow in a polarized fashion and have a characteristic, asymmetric shape during part or all of their life cycle. In higher eukaryotes, polarized growth is vital to many biological phenomena, such as nerve impulse transmission, chemotaxis and epithelial solute transport. Despite its importance, our understanding of how cells establish and maintain polarity is rudimentary.

In the budding yeast *Saccharomyces cerevisiae*, a number of genes have been identified that affect polarized growth (reviewed in Chant 1994; Chenevert, 1994). Both the budding and mating processes require a period of asymmetric growth and many of the genes that regulate these processes encode proteins of known function, such as protein kinases and small GTPases of the Ras superfamily. Interestingly, homologs of a number of these genes have also been implicated in the control of cell polarity in the fission yeast *Schizosaccharomyces pombe*, which is only distantly related to the budding yeasts (Chang *et al.*, 1994; Miller and Johnson, 1994; Nurse 1994). These include genes encoding a rho-like GTPase, *CDC42* (*cdc42* in fission yeast) and several factors that appear to regulate *CDC42*, including a guanine-nucleotide exchange factor, *CDC24* (*scd1/ral1*), a geranyl geranyltransferase, *CDC43* (*cwg2*) and an SH3 domain-containing protein, *BEM1* (*scd2/ral3*).

In *S.cerevisiae*, disruption of *CDC42* function results in non-viable, large, round, unbudded cells (Adams *et al.*, 1990). Expression of constitutively active alleles of *CDC42*, encoding GTPase-deficient forms of the protein, results in a lethal phenotype, characterized by large cells with elongated or multiple buds (Ziman *et al.*, 1991). In *S.pombe*, *cdc42* is required for vegetative growth and gene disruption results in arrest as small, round, dense, cells (Miller and Johnson, 1994). Unlike budding yeast, expression of constitutively active *cdc42* alleles in *S.pombe* is not lethal, but instead results in an abnormal morphological phenotype of large, misshapen cells (Miller and Johnson, 1994). Thus, despite certain differences, evidence from both budding and fission yeast indicates an important role for Cdc42 in morphogenesis.

Several Cdc42 homologs have also been identified in higher eukaryotes, including *Caenorhabditis elegans*, *Drosophila melanogaster* and *Homo sapiens*. The human Cdc42 protein is ~85% identical to its homologs in yeast and is closely related to mammalian Rac1 and RhoA (Munemitsu *et al.*, 1990; Shinjo *et al.*, 1990). These latter enzymes are known to regulate actin reorganization in response to various extracellular stimuli (Ridley and Hall 1992; Ridley *et al.*, 1992). As such reorganization directly affects cell shape, these findings suggest that the basic molecular machinary underlying asymmetric cell growth is broadly conserved among all eukaryotes.

How does Cdc42 regulate polarized growth? Recently, a rat brain protein kinase termed p65^{pak} was isolated by virtue of its ability to bind to Rac1 and Cdc42Hs. *In vitro*, p65^{pak} binds specifically to GTP-bound Rac1 and Cdc42 (Manser *et al.*, 1994). As a result of this association, the p65^{pak} kinase autophosphorylates on serine and threonine residues and becomes activated towards exogenous substrates. Thus, p65^{pak} may be an important effector for Rac1 and Cdc42 in actin reorganization pathways in mammalian cells. In budding yeast, a p65^{pak} homolog termed *STE20* has been identified as a member of the

mating pathway (Leberer *et al.*, 1992; Ramer and Davis, 1993). *STE20* is required not only for mating but also for filamentous growth of diploids (Liu *et al.*, 1993), which represents an extreme example of polarized growth. Like $p65^{pak}$, Ste20p binds to Cdc42p *in vitro* (Manser *et al.*, 1994). Genetic evidence suggests that Ste20p acts at the level of, or just distal to, the mating pheromone receptor (Leberer *et al.*, 1992) and may therefore not require any other protein kinases for activation. Thus, the Ste20p-like enzymes may be among the first kinases activated in certain signaling systems. As both the mating and budding processes in yeast entail asymmetric cell growth, it is possible that Ste20p or other related kinases form part of the signaling apparatus through which cell polarity changes occur. In fact, Cdc42p localizes to sites of polarized growth in both budded cells and mating projections (Ziman *et al.*, 1993).

In this communication, we identify and characterize one of at least two Ste20/$p65^{pak}$-like protein kinases from fission yeast, termed Pak1Sp. Unlike *STE20* in *S.cerevisiae*, the *S.pombe* $pak1^+$ gene is required for vegetative growth. However, when expressed in *STE20*-null *S.cerevisiae* cells, $pak1^+$ partially restores mating function. We further show that *S.pombe* Pak1p binds to Cdc42p *in vitro* and *in vivo*, and this binding is mediated through the effector domain of Cdc42p. Furthermore, overexpression and gene disruption experiments show that Pak1p has marked effects on cell shape, actin localization and mating. Taken together, these findings suggest that Pak1p plays a role in mediating cell polarity and mating in fission yeast, and may be an effector for Cdc42p or a related GTPase. These findings also further underscore the conserved nature of the molecular machinary controlling cell shape in eukaryotes.

Results

Isolation of a $p65^{pak}$/STE20-like cDNA from S.pombe

We designed oligonucleotide primers based on conserved amino acid sequences in $p65^{pak}$ and Ste20p. We used these oligonucleotides to amplify an ~500 bp band from a *S.pombe* cDNA library by PCR. Following reamplification, the product was subcloned into pUC19 and the sequences of inserts from 10 independent colonies were determined. One insert encoded a potential kinase fragment similar, but not identical, to $p65^{pak}$ and Ste20p. We named this gene $pak1^+$ because of its high degree of sequence homology to rat $p65^{pak}$ and because this protein, like its mammalian homolog, appears to interact with a p21 GTPase (see below). In order to isolate the full-length cDNA, we then used this insert to probe the *S.pombe* library. A total of five independent clones were isolated in this manner. Four of the cDNAs contained internal sequences identical to the PCR product. One cDNA contained a related insert ($pak2^+$) which also encodes a putative homolog of $p65^{pak}$ and Ste20p (not shown).

The $pak1^+$ sequence potentially encodes a protein of 72 kDa (Figure 1). We believe the cDNA is full-length because the transcript size (3.2 kb) is similar to the cDNA size (2.9 kb) (data not shown) and the presumptive initiation ATG meets the criteria for a Kozak consensus site (A/GNNATGG) (Kozak, 1987) and is preceeded by an in-frame stop codon. The 3′ untranslated region contains a polyadenylylation signal (AATAAA) and ends in a polyadenylate tract. The putative protein kinase catalytic domain is 71 and 67% identical to those of Ste20p and $p65^{pak}$, respectively. This domain is also closely related to a second subgroup of kinases, including human Gck (Katz *et al.*, 1994), human Mst1 (Creasy and Chernoff, 1995) and *S.cerevisiae SPS1*, which is involved in spore formation in budding yeast (Friesen *et al.*, 1994) (Figure 2A and B). The amino-terminus contains a region homologous to the Rac1/Cdc42 binding domains of Ste20p, $p65^{pak}$ and $p120^{ack}$ (Figure 2C).

$Pak1^+$ encodes a serine/threonine protein kinase

To assess the enzymatic activity of Pak1p, we examined the ability of Pak1p to autophosphorylate in an *in situ* kinase assay. *Schizosaccharomyces pombe* cells were transformed with the expression vector pREP3X-pak1, which uses the thiamine-repressable nmt^+ promoter to regulate expression (Maundrell, 1990; Forsburg, 1993). Protein extracts from control (thiamine plus) or Pak1p-expressing (thiamine minus) *S.pombe* were separated by SDS–PAGE, transferred to an PVDF membrane and renatured using guanidine chloride. The membrane was then incubated in protein kinase buffer in the presence of [γ-^{32}P]ATP, washed and exposed to film. Comparison of the *in situ* kinase blot with an anti-hemagglutinin (HA) immunoblot of the same protein samples indicates that an autophosphorylating activity comigrates with epitope-tagged Pak1p (Figure 3A, lanes 4 and 8). To exclude the possibility that the observed phosphorylation is due to a co-migrating kinase, we carried out the same experiment with a kinase-inactive form of this enzyme. The results of this control (Figure 3A, lanes 2 and 6) indicate that Pak1p accounts for the phosphorylating activity. The autophosphorylation sites on Pak1p are predominantly on serine residues (Figure 3B). This pattern is similar to that found for autophosphorylated rat $p65^{pak}$ (Manser *et al.*, 1994).

Pak1p binds to activated Cdc42p

As Pak1p contains an amino-terminal sequence motif similar to the Rac1/Cdc42 binding domain of $p65^{pak}$, we tested whether Pak1p could bind to *S.pombe* Cdc42p *in vitro*. A GST fusion protein encoding Cdc42p was produced in *Escherichia coli*, purified over glutathione–agarose and loaded with either GDP or a non-hydrolyzable form of GTP (GTP-γS). Protein extracts from Pak1p-induced cultures of *S.pombe* were incubated with the glutathione–agarose conjugates, washed and bound proteins analyzed by immunoblotting. The results indicate that Pak1p interacts preferentially with activated forms of Cdc42p (Figure 4A).

We also carried out the reciprocal binding experiment, using immobilized GST–Pak1 to capture Cdc42p from cell extracts. *Schizosaccharomyces pombe* cells overexpressing either Myc-tagged wt Cdc42p, inactive $Cdc42p^{T17N}$ or effector domain mutant $Cdc42p^{T35A}$ (Table I), were incubated with immobilized glutathione-*S*-transferase (GST) fusion protein containing the presumptive p21 binding domain (PBD) from Pak1p. As before, the results of this experiment show a preferential interaction between active Cdc42p and the Pak PBD (Figure 4B). In addition, a lack of binding of $Cdc42p^{T35A}$ mutant protein to Pak1p shows

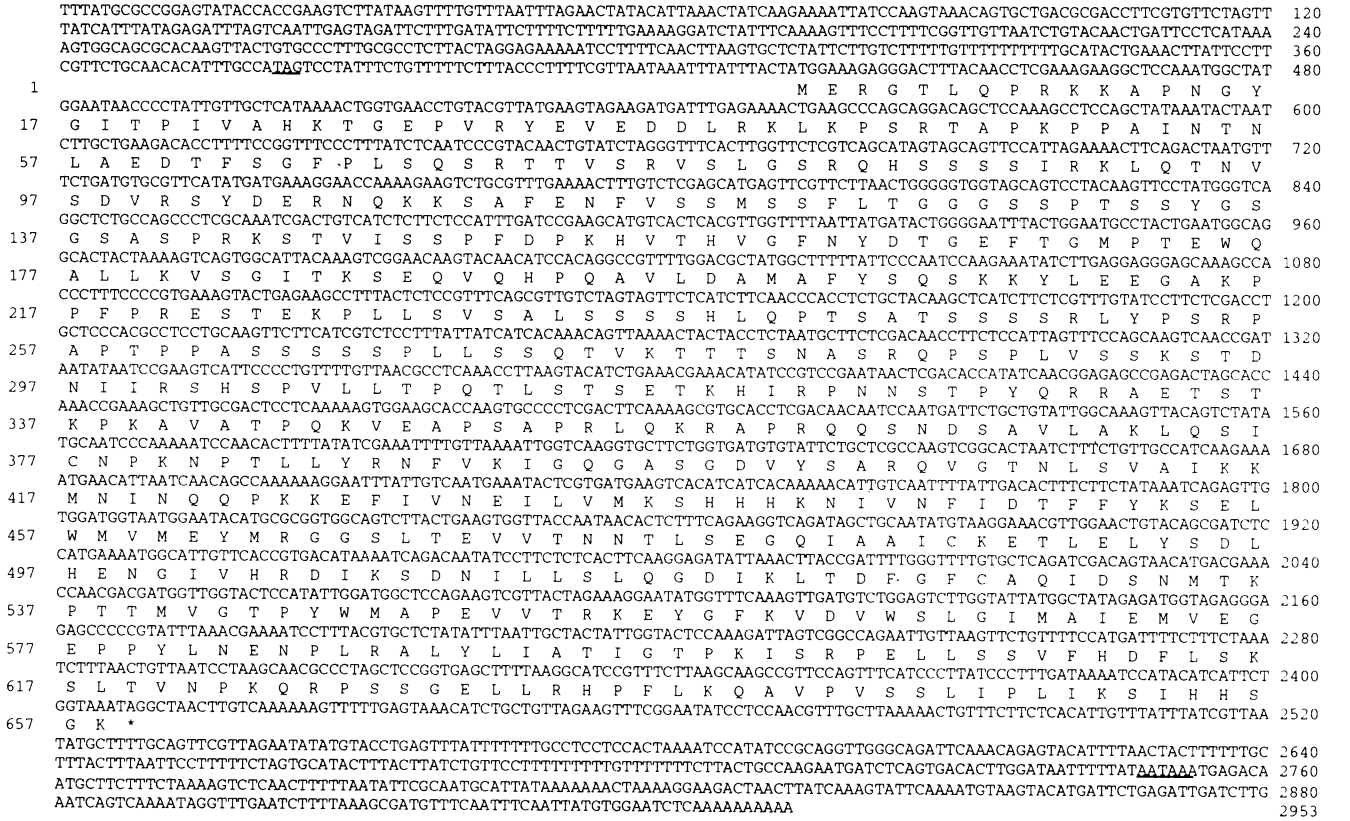

Fig. 1. Sequence of *pak1*$^+$and its protein product. The DNA sequence of the *S.pombe pak1*$^+$ gene and its predicted amino acid sequence. The DNA sequence is numbered at right, the amino acids are numbered at left; amino acids appear in single-letter code beneath nucleotides. The termination codon is marked with a star and the polyadenylylation signal (AATAAA) and an upstream stop codon (TAG) are underlined. These sequence data are available from EMBL/GenBank/DDBJ under accession number U22371.

that the effector domain of Cdc42p is essential for this interaction.

Similar results were obtained using an *in vivo* interaction-trap to evaluate Pak1p–Cdc42p interactions (Table II). In this experiment, a lexA–Pak1p construct was co-expressed in *S.cerevisiae* with various forms of activation domain–cdc42 fusions. All Cdc42p constructs contained a C189S mutation, which eliminates the isoprenylation site. This modification was included to relieve toxicity and to promote entry into the nucleus. Two reporters of interaction were used, lacZ activity and ability to grow in the absence of leucine. Both assays gave similar results. Pak1p preferentially binds to activated Cdc42p (Table II, compare lines 2 with 3) and binding is eliminated by the Cdc42p^{T35A} effector domain mutation (Table II, compare lines 3 with 5). Pak1p interacts slightly with dominant-negative Cdc42p^{T17N}. A bait plasmid encoding an irrelevant protein (RPB7) (McKune *et al.*, 1993) did not interact with Cdc42p.

Phenotypes of pak1 and cdc42 overexpression

Since the proteins encoded by *pak1*$^+$ and *cdc42*$^+$ are biochemically linked, we asked whether they are also genetically linked. Overexpression of Pak1p was not able to restore viability to Δ*cdc42* cells (data not shown). Overexpression of either wild-type or inactive (K415,416R) Pak1p, or activated (Q61L) or dominant-negative (T17N) Cdc42p alone resulted in viable cells (Figure 5). However, co-expression of *cdc42*^{T17N} or *cdc42*^{Q61L} with *pak1*$^+$ was lethal (Figure 5). Similarly, these combinations of either form of mutant *cdc42* and inactive *pak1* also failed to grow, suggesting that the lethality associated with overexpression of these proteins is not a function of the kinase activity of *pak1*.

When overexpressed in wild-type cells, *pak1*$^+$ caused a markedly abnormal morphological phenotype, characterized by enlarged, misshapen cells (Figure 6B; Table III). This morphological phenotype is unique and generally not the same as that seen with the Q61L-activated *cdc42* allele (Miller and Johnson, 1994), except for the rare large rounded cells. Overexpression of *cdc42*^{T17N} alone results in a shortened, rounded morphology (Figure 6C). Combined expression of *cdc42*^{T17N} and either wild-type or *pak1*^{K415,416R} leads to a lethal growth defect, which we term a 'synthetic overdose' phenotype. In both cases, the morphology of the dead and dying cells is a combination of the two individual morphologies along with a new morphology (Figure 6D). These data suggest that overexpression of *pak1*$^+$ does not suppress the T17N phenotype and may exacerbate a growth defect. Overexpression of *pak1*^{K415,416R} alone leads to rounded cells that are morphologically different from wild-type *pak1*-expressing cells. Actin patches are mislocalized to the periphery of the rounded up cells, suggesting that overexpression of inactive Pak1p kinase interferes with polarized growth (Figure 7B and C). Overexpressing *pak1*^{K415,416R} in combination with either *cdc42*^{T17N}or *cdc42*^{Q61L} leads to a lethal growth defect; in both cases, the morphology of the dead and dying cells look more like the *cdc42* mutant

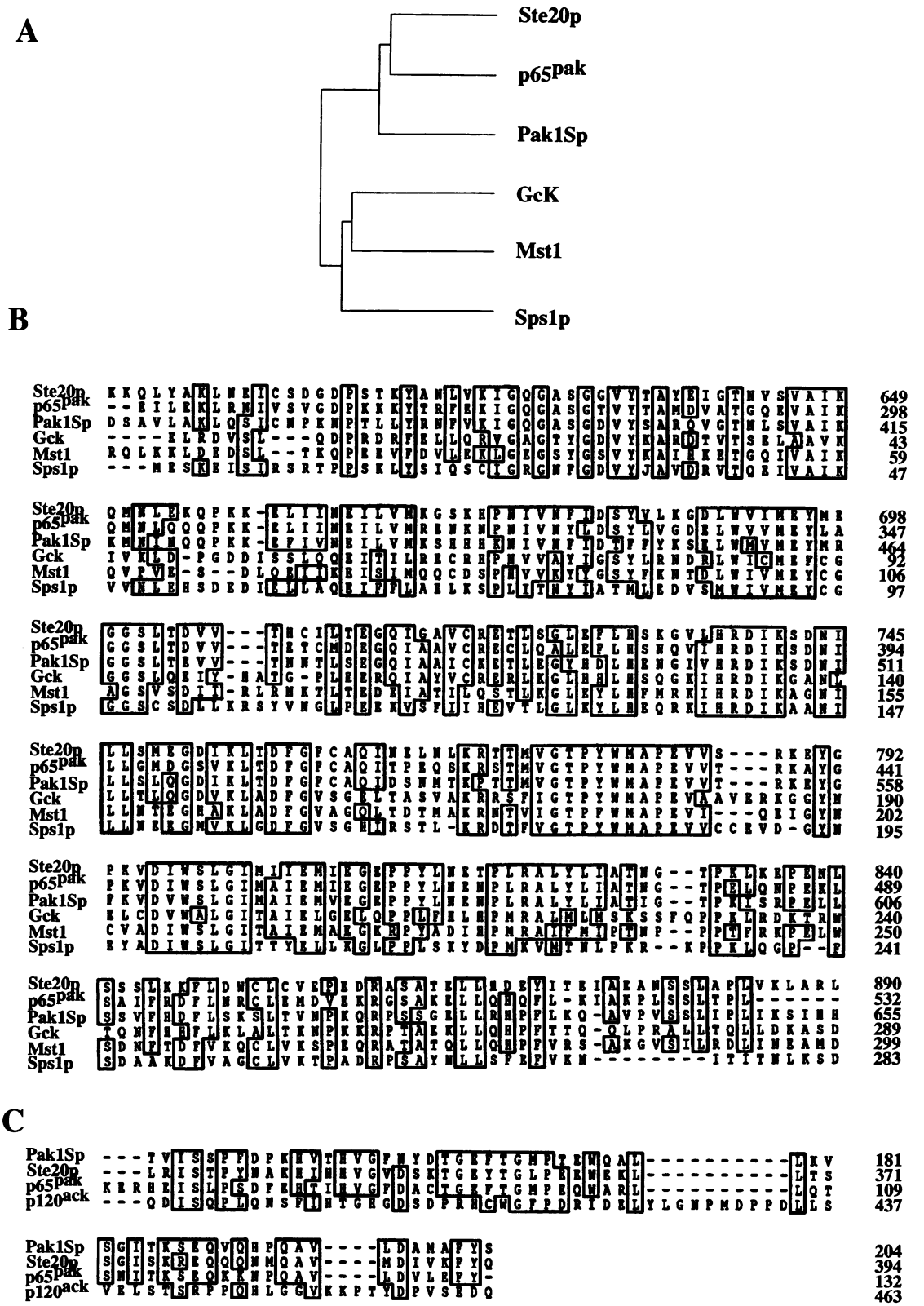

Fig. 2. The STE20/pak family of protein kinases. (**A**) The amino acid sequences of *S.cerevisiae* Ste20p (Leberer *et al.*, 1992), rat p65^{pak} (Manser *et al.*, 1994), *S.pombe* Pak1Sp, human Mst1 (Creasy and Chernoff, 1995), human GC kinase (Katz *et al.*, 1994) and *S.cerevisiae* Sps1p (Friesen *et al.*, 1994) were aligned using the Pileup algorithm of the GCG program. The dendrogram represents the degree of homology between each sequence. (**B**) The catalytic domains of each kinase are aligned using the Prettyplot function of the GCG program. (**C**) Prettyplot comparison of the putative PBDs of *S.cerevisiae* Ste20p, rat p65^{pak}, *S.pombe* Pak1Sp and human p120^{ack} (Manser *et al.*, 1993). Amino acid positions are given on the right.

morphologies, again suggesting that suppression of *cdc42* mutant phenotypes is not seen.

Loss of pak1$^+$ is lethal

In order to test further for function of the *S.pombe pak1$^+$* gene, part of the cDNA encoding the kinase domain of Pak1p was replaced with the *S.pombe ura4$^+$* gene. A diploid *S.pombe* strain was constructed and transformed with the *pak1::ura4$^+$* fragment. Stable uracil-prototrophic colonies were isolated and transformants containing one disrupted allele of *pak1* and one wild-type copy were identified by DNA–DNA hybridization analysis (data not shown).

Sporulation of the diploid strain was induced and tetrads were dissected (data not shown). All tetrads (16 dissected) resulted in only two viable spores and these were uracil auxotrophs. This indicates that the viable spores carry the *pak1$^+$* wild-type gene, and that the disruption of *pak1* leads to inviable spores. We examined the phenotype of the inviable spores microscopically and found that most spores underwent one or two divisions that resulted in very small and round cells that stuck together. This phenotype is similar to that observed with a *cdc42* null mutation (Miller and Johnson, 1994).

In order to characterize further the phenotype of cells deleted for a functional *pak1* product, we carried out a promoter shut-off experiment. The diploid strain carrying the *pak1* disruption was transformed with either a full-length pREP3X-*pak1* construct (leu$^-$ selection) or a *pak1* truncation lacking 276 amino-terminal amino acids (FN–*pak1*). Although similar in structure to a constitutively active *STE20* allele (Ramer and Davis, 1993), the FN–*pak1* construct encodes an unstable form of Pak1 with reduced kinase activity (not shown). As a control, the diploid strain was also transformed with the vector alone. Sporulation was induced and spores were germinated on medium lacking uracil (to select for the disruption), leucine (to select for the plasmid) and thiamine (to induce expression from the *nmt1$^+$* vector). Only spores carrying either the full-length or the truncated version of *pak1* were able to form viable colonies (Figure 8A). Spores carrying

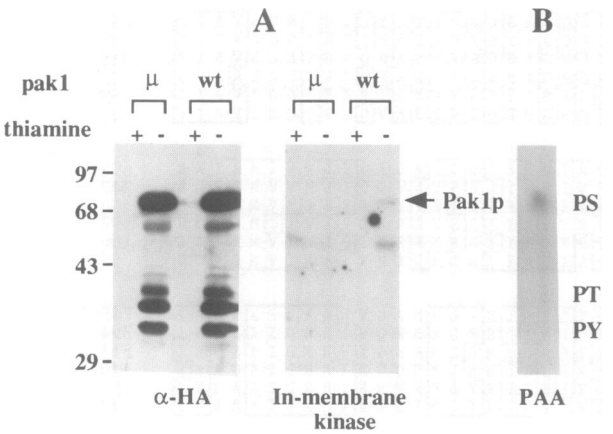

Fig. 3. Protein kinase activity of Pak1p. *Schizosaccharomyces pombe* was transformed with expression vectors encoding either catalytically inactive (μ) or wild-type (wt) Pak1p. Ten ml of cells were grown to mid-log phase in either the absence or presence of thiamine. Protein extracts were analyzed for the presence of Pak1p (**A**, α-HA) and for protein kinase activity (in-membrane kinase). Molecular weight markers are indicated. The radiolabeled band that comigrates with Pak1p in (A) (arrow) was then excised and subjected to phosphoamino acid analysis (**B**).

Table I. Cdc42p and Pak1p mutants and their activities

Protein	Activity
Cdc42	wild-type
Cdc42 G12V	constitutively active, GTPase deficient
Cdc42 T17N	dominant negative
Cdc42 T35A	effector domain mutant
Cdc42 Q61L	constitutively active, GTPase deficienct
Pak1	wild-type
Pak1 K415,416R	dominant negative, kinase inactive

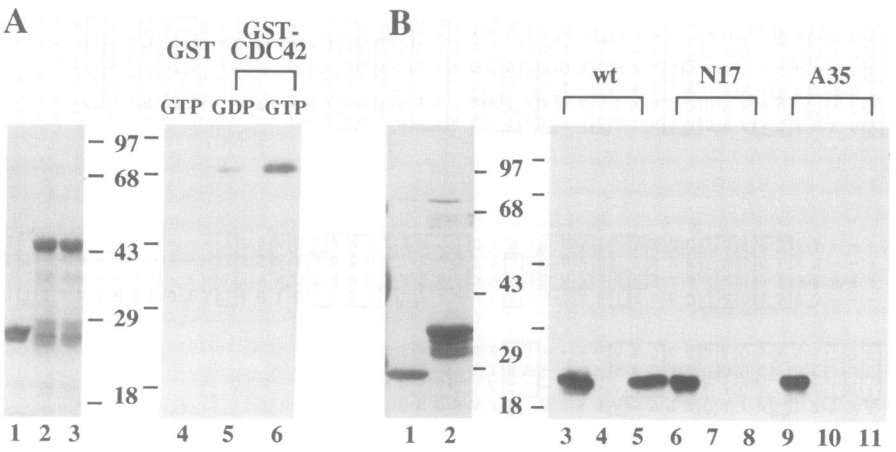

Fig. 4. *In vitro* interactions between Cdc42p and Pak1p. (**A**) *S.pombe* was transformed with an expression vector encoding HA-epitope-tagged Pak1p. Ten ml of cells were grown to mid-log phase in the absence of thiamine and protein extracts from these cells were incubated with either GST alone (lane 4), GDP-loaded GST–Cdc42 (lane 5) or GTP-loaded GST–CDC42 (lane 6). Signals were detected by anti-HA immunoblot. Coomassie stains of GST, GDP-loaded GST–CDC42 and GTP-loaded GST–CDC42 are shown in lanes 1, 2 and 3, respectively. (**B**) *Schizosaccharomyces pombe* was transformed with an expression vector encoding either Myc-epitope-tagged wt Cdc42p, Cdc42p^{T17N} or Cdc42p^{T35A}. Ten ml of cells were grown to mid-log phase in the absence of thiamine, and protein extracts from these cells were either analyzed directly (lanes 3, 6 and 9) or incubated with either GST (lanes 4, 7 and 10) or GST–PAK1 PBD (lanes 5, 8 and 11). Signals were detected by anti-Myc immunoblot. Coomassie stains of GST and GST–PAK1 PBD are shown in lanes 1 and 2, respectively. Molecular weight markers are indicated.

Table II. Protein complex formation detected by interaction trap

LexA fusion	Activation domain fusion	Growth on leu⁻ medium	β-galactosidase (units ± SD)
Pak1	–	–	1.2 ± 0.2
Pak1	cdc42	+	10.1 ± 2.8
Pak1	cdc42 G12V	++++	55.0 ± 8.1
Pak1	cdc42 T17N	–	4.1 ± 0.7
Pak1	cdc42 G12V/T35A	–	1.1 ± 0.3
RPB7	cdc42 G12V	–	1.5 ± 0.4

LexA fusion protein baits in pEG202 were co-transformed with the indicated activation domain fusion vectors, plus the *lacZ* reporter pSH18-34 into *S.cerevisiae* EGY48. Protein expression was induced by growing the transformants on galactose-containing medium. Interactions were detected by assaying for degree of growth on leu⁻ medium and by measuring β-galactosidase activity. The indicated activities represent averages from three independent transformants. SD, standard deviation.

the vector alone did not form colonies on medium lacking uracil and leucine, but were able to form colonies on plates providing uracil. These colonies were uracil auxotrophs, indicating that the spores contained the wild-type *pak1*$^+$. Thus, when the promoter is fully induced, both full-length and truncated forms of *pak1*$^+$ are able to rescue the null allele.

Single colonies of transformants with either the full-length or truncated *pak1*-pREP3X construct had different growth rates. When these transformants were streaked onto medium containing thiamine, colonies could form from cells containing the full-length construct, but not from cells carrying the truncated construct (Figure 8A). The *nmt1*$^+$ promoter is known to be slightly leaky under repressed conditions and this suggested that there was a difference in the ability of the two clones to rescue. We characterized this phenotype further by growing the cells in liquid culture. Cells were grown in the absence of thiamine to mid-log phase, diluted and either reinoculated in medium lacking thiamine or into medium containing 20 μM thiamine to repress expression. We harvested the cells after 24 h in inducing or repressing conditions, fixed them in methanol–glacial acidic acid and stained the nuclei with DAPI. As shown in Figure 8B, *pak1*-null cells overexpressing full-length *pak1*$^+$ show some degree of abnormal cell morphology; they are round and enlarged compared with wild-type cells (Figure 8A). Upon addition of thiamine these cells remain viable, but divide at smaller cell size (Figure 8C). Thus, overproduction of the full-length *pak1*$^+$ induces an abnormal phenotype. The culture overexpressing the truncated version of *pak1* contains a high number of cells with very abnormal cell morphology (Figure 8D). Cells are quite small and very round and growth rate and viability are reduced relative to the wild type. Upon addition of thiamine the cells arrest with a septum and two nuclei (Figure 8E). This phenotype is very similar to that seen when the disrupted spores germinate. Western blot analysis shows that the truncated protein is much less abundant than the full-length protein (data not shown). This is most likely due to greater instability of the truncated protein than to different expression levels of the *nmt1*$^+$ promoter.

Although superficially similar to 'cut' or 'mitotic catastrophe' phenotypes, both nuclei appear to be equally stained with DAPI and well separated by the septum, and

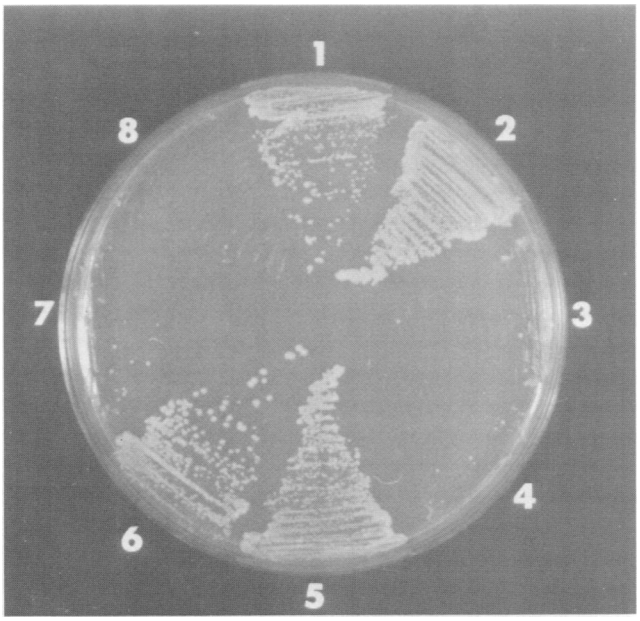

Fig. 5. Growth of wild-type haploid cells containing plasmid-borne *pak1* and *cdc42* alleles under the control of the thiamine-repressible *nmt1*$^+$ promoter. Cells were patched from selective medium plates plus thiamine to thiamine⁻ plates then serially streaked twice to thiamine⁻ plates. Combinations of expressed alleles are as follows: 1, *pak1*^{K415,416R} and control vector pREP3X; 2, *pak1*$^+$ and control vector pREP4X; 3, *pak1*^{K415,416R} and *cdc42*^{T17N}; 4, *pak1*$^+$ and *cdc42*^{T17N}; 5, *cdc42*^{T17N} and control vector pREP3X; 6, *cdc42*^{Q61L} and pREP3X; 7, *pak1*$^+$and *cdc42*^{Q61L}; 8, *pak1*^{K415,416R} and *cdc42*^{61L}.

there is no evidence for failure of nuclear division or cutting of the nucleus. Therefore, we consider this phenotype distinct. It is quite reminiscent of the *cdc42* phenotype (Miller and Johnson, 1994) or the *orb* phenotypes (Snell and Nurse, 1994).

Pak1$^+$ *suppresses the mating defect of a ste20 null allele in budding yeast*

To assess the ability of *pak1*$^+$ to suppress the mating defect caused by a deletion in *STE20*, a *S.cerevisiae* strain lacking *STE20* (YEL-33-7-3B, Table VI) was transformed with a galactose-inducible expression vector, bearing either no insert (pYES2), *STE20* (pFLC1; Ramer and Davis, 1993) or cDNA encoding full-length Pak1p (pYES2-*pak1*$^+$). A patch mating test demonstrated that *pak1*$^+$ could suppress the mating defect (Figure 9). Mating did not occur when the cells were grown in glucose indicating that mating was dependent on expression of *pak1*$^+$ (data not shown). A quantitative mating assay demonstrated that *pak1*$^+$ allows mating at 10–30% of the level of *STE20* (Table IV). Suppression is specific for *STE20*, since *pak1*$^+$ was unable to suppress the mating defect of strains containing deletion in either *STE11* or *STE5* (Figure 9). Suppression of *STE20* was not observed when the cells were plated on glucose-containing medium, indicating that the growth was due to Pak1p production (data not shown).

Pak1 function is required for mating in fission yeast

As *pak1*$^+$ function is required for viability, we assessed its effects on mating and sporulation by overexpressing catalytically inactive Pak1 in the context of a wild-type

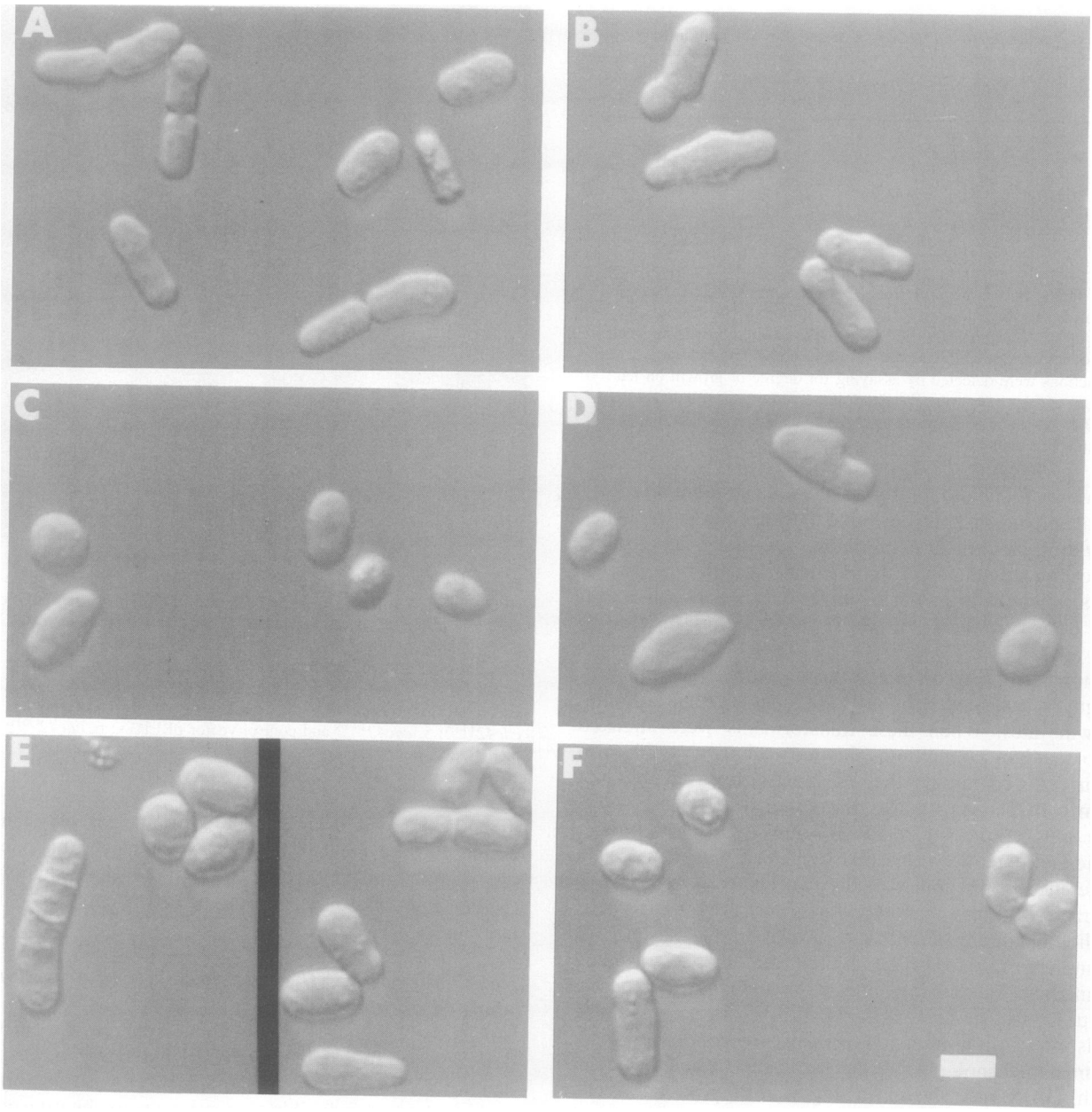

Fig. 6. Overexpression phenotypes of *pak1* and *cdc42* alleles. Shown are photomicrographs of wild-type haploid cells containing the following combinations of plasmid-borne *pak1* and *cdc42* alleles under the control of the thiamine-repressible *nmt1*$^{+}$ promoter: (**A**) two control vectors pREP3X and pREP4X, (**B**) *pak1*$^{+}$ and pREP4X, (**C**) *ccdc42*^{T17N}and pREP3X, (**D**) *pak1*$^{+}$ and *cdc42*^{T17N}, (**E**) *pak1*^{K415,416R} and pREP4X, (**F**) *pak1*^{K415,416R} and *cdc42*^{T17N}. Size bar is 6 μm.

genetic background. Overexpressing either *pak1*^{K415,416R} or *cdc42*^{T17N} severely reduced mating efficiency, while the wild-type genes did not (Table V). Cells grown in the presence of thiamine showed nearly normal mating, indicating that the mating defect is specifically caused by expression of the mutant genes. Expression of *pak1*^{K415,416R} or *cdc42*^{T17N} reduced the vegetative growth rate by only ~20% by (not shown), indicating that the observed mating defect is not simply a reflection of toxicity.

Discussion

In this paper, we characterize the first of at least two p65^{pak}/Ste20p-like protein kinases in the fission yeast *S.pombe*. We show that *pak1*$^{+}$ encodes a serine/threonine-specific protein kinase which is capable of autophosphorylation, as are Ste20p and rat p65^{pak}. Expression of *pak1*$^{+}$ is essential for growth and cells lacking Pak1p die as small, round cells, a phenotype very similar to that of *S.pombe cdc42*-null cells, suggesting that Pak1p may function in the same morphological pathway as Cdc42p. Cdc42p is a Rho GTPase homolog which has been implicated in both budding and fission yeast and in mammalian systems as functioning in actin localization. Activated (GTP-bound) Cdc42p has been shown in other systems to interact with Ste20p-like kinases. We have obtained both biochemical and genetic evidence, including Pak1p binding to activated Cdc42p *in vitro*, a two-hybrid protein interaction *in vivo* and a synthetic overdose phenotype between Pak1p alleles and Cdc42p mutant

Table III. Morphological phenotypes associated with overexpression of wild-type and mutant alleles[a]

Expressed alleles		Phenotypes (%)[b]			
pak1	*cdc42*	Normal rod	Irregular	Small round	Large rounded
–	–	86.0	0.0	14.0	0.0
WT	–	47.5	44.0	7.5	1.0
–	*T17N*	19.0	0.0	81.0	0.0
WT	*T17N*	24.0	10.5	44.0	21.5
K415,416R	–	63.0	7.0	0.0	30.0
K415,416R	*T17N*	66.5	4.0	25.0	4.5
–	*Q61L*	42.0	43.0	15.0	0.0
WT	*Q61L*	43.5	41.0	14.0	0.0
K415,416R	*Q61L*	26.5	48.0	25.0	0.0

[a]Mid-log cells containing the indicated plasmid-borne alleles under the control of the *nmt1*$^+$ thiamine-repressible promoter were grown to mid-log phase in thiamine-containing medium. The cells were then washed and serially subcultured twice without thiamine. Dashes indicate a control vector. [b]Two-hundred cells from each culture were examined using Hoffman modulation optics and assigned to morphological categories based on size and shape. 'Small round' cells were <6 μM in diameter. 'Large rounded' cells have a short axis >6 μM and included both spherical and ovoid cells. The 'irregular' category seen with *pak1*$^+$deviated from the wild-type uniform rod morphology having swellings, bends and/or strictures and tended to be longer than average wild-type cells. The 'irregular' category seen with *cdc42*^{Q61L} cells included elongated (>20 μm) cells as well as enlarged, round, misshapened cells.

alleles, suggesting a functional interaction between *S.pombe* Pak1p and Cdc42p or a similar GTPase. These results, taken together with the morphological and actin localization phenotype observed in cells overexpressing an inactive form of *pak1*, also suggest that *pak1*$^+$ may play a role in generating and maintaining cellular polarity. This kinase may also function in mating, as evidenced by the mating defect observed in cells overexpressing inactive *pak1*, as well as the ability of wild-type *pak1*$^+$ to suppress the mating defect of *STE20* deletions in budding yeast.

The interaction between Pak1p and Cdc42p (as detected by two-hybrid interaction-trap) is eliminated by a Cdc42p effector domain mutation, suggesting that Pak1p is a downstream effector of Cdc42p function. Pak1 does not, however, suppress the lethality associated with *cdc42* disruption, nor does overexpression of *pak1*$^+$ suppress the morphologic and growth defects associated with expression of inactive (N17) *cdc42*. Indeed, such co-expression results in cell death. These data can be interpreted in at least three ways: (i) Pak1p requires activation by Cdc42p, thus, overexpression of Pak1p in the absence of activated Cdc42p is unproductive and perhaps even toxic; (ii) Pak1p is not the sole downstream effector for Cdc42p; or (iii) Pak1p is not an effector for Cdc42p, but is instead an effector for a related GTPase. Although we cannot at this time definitively distinguish between these possibilities, we favor the second and believe that the preponderance of biochemical and genetic evidence supports the interpretation that Pak1p interacts with Cdc42p *in vivo*, and has a role in mediating the effects of Cdc42p on polarized growth and mating.

In budding yeast, Cdc42p is necessary for establishing the polarity required for both budding and mating. The *STE20* gene in budding yeast operates in the mating pathway, as an upstream component of the MAP kinase signal transduction pathway that mediates pheromone response. *STE20* mutants are sterile, a phenotype rescued by overproduction of Pak1p and overexpression of an inactive mutant form of Pak1p inhibits mating in *S.pombe*. We therefore believe that *pak1*$^+$ encodes a true Ste20p homolog, which also functions in the *S.pombe* mating response pathway. However, other mutants in this pathway cause sterility, whereas *pak1*$^+$ is essential for viability.

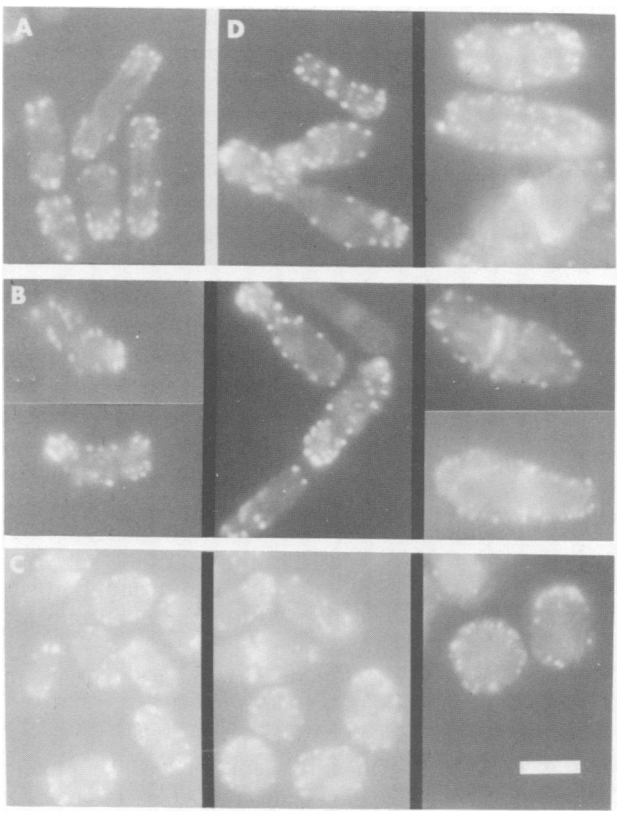

Fig. 7. Actin localization in strains overexpressing *pak1* alleles. Formaldehyde-fixed cells expressing the following alleles were stained with rhodamine-conjugated phalloidin: (**A**) wild-type cells containing control vector, (**B**) *pak1*$^+$, (**C**) *pak1*^{K415,416R}, (**D**) *cdc42*^{Q61L}. Size bar is 6 μm.

Thus, we conclude *pak1*$^+$ is involved in an essential morphology pathway distinct from the mating pheromone response, although it may be involved in both.

A number of morphological mutants define non-essential genes which may affect mating as well as polarity, such as *ras* mutants (Fukui *et al.*, 1986; Nadin-Davis *et al.*, 1986; Xu *et al.*, 1994) or mutants in the *ral/scd* genes (Fukui and Yamamoto, 1988; Chang *et al.*, 1994). Clearly these genes affect polarity, but they are not

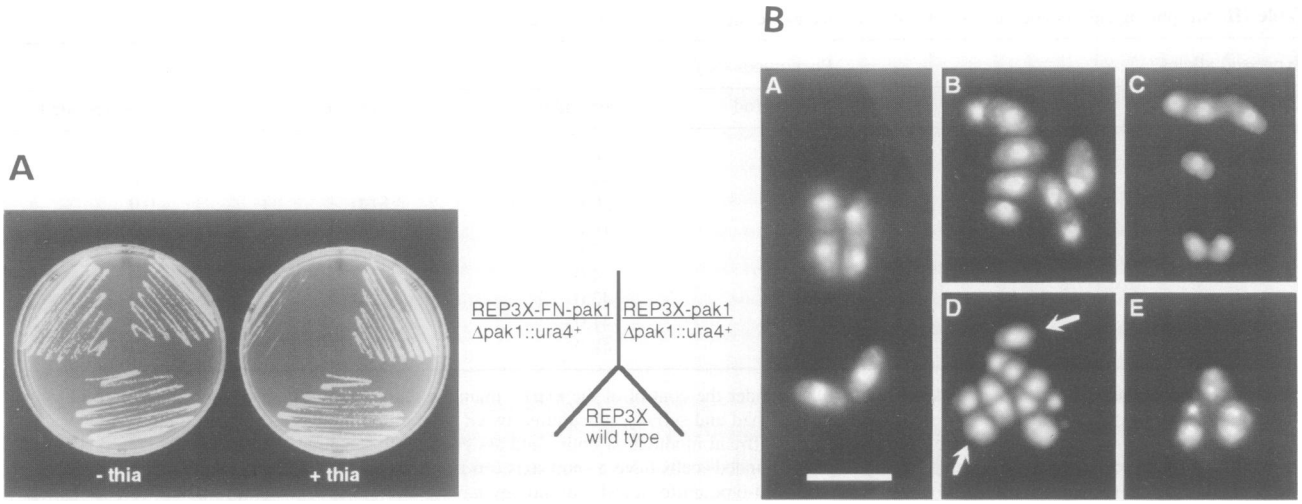

Fig. 8. Characterization of *pak1*-disrupted *S.pombe*. The diploid strain carrying the pak1 disruption was transformed with either a full-length pREP3X-pak1 construct or a *pak1* truncation lacking 276 amino-terminal amino acids (FN-pak1), followed by leu$^-$ selection. (**A**) The cells, plus a wild-type control transformed with empty vector, were grown in either the presence or absence of thiamine. (**B**) Morphologies of pak1-disrupted cells transformed with either full-length pREP3X-pak1 grown in the absence (B) or presence (**C**) of thiamine or FN-pak1 grown in the absence (**D**) or presence (**E**) of thiamine. Control wild-type cells are shown in (A).

required for cell viability. Other genes which are essential for polarity, *cwg2*$^+$ and *cdc42*$^+$, have homologs in budding yeast (*CDC43* and *CDC42*, respectively; Diaz *et al.*, 1993; Miller and Johnson, 1994). There is now evidence linking genes such as *ral1*$^+$/*scd1*$^+$ and *ral3*$^+$/*scd2*$^+$ with *cdc42*$^+$ (Chang *et al.*, 1994) and by extension, possibly with *pak1*$^+$ as well. Mutations in a diverse number of *S.pombe* genes lead to morphological phenotypes similar to *pak1* phenotypes (Nurse, 1994). Some of these are likely to affect cell wall structure directly, such as such as *cwg1*$^+$ (Ribas *et al.*, 1991a,b). A number of kinases and phosphatases also affect cell morphology, including protein kinase C (PKC) homologs (Toda *et al.*, 1993). Intriguingly, PKC is involved in a MAP kinase pathway in budding yeast that influences cell wall integrity (Levin and Bartlett-Heubusch, 1992; Paravicini *et al.*, 1992). A collection of round mutants, *orb1–orb5*, was recently described by Snell and Nurse and one of these encodes an alpha subunit of casein kinase II (Snell and Nurse, 1994).

That more than one Ste20p homolog exists in fission yeast is consistent with a role for Ste20p-like protein kinases in different signaling pathways. At least four MAP kinase pathways have been identified in budding yeast, involved in diverse cell functions ranging from mating to osmoregulation to cell-wall structure and proliferation. Fewer complete pathways have been dissected in fission yeast, although individual components of these pathways have been identified. It therefore seems likely the *pak1*$^+$ gene may define another potential MAP kinase pathway linking cell polarity/morphology and cell growth. By analogy to the situation in budding yeast, where Ste20p lies atop the mating signal cascade, it is possible that Pak1p or Pak2p activates a MAP kinase pathway by phosphorylating Byr2p or a similar kinase. Identification of the substrates of Pak1p, therefore, promises to extend our understanding of the role of these kinases in eukaryotic cell growth.

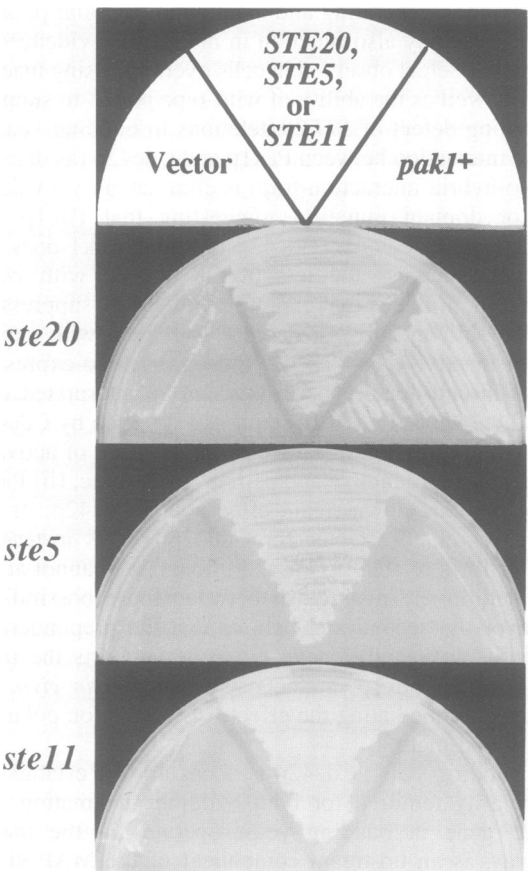

Fig. 9. Trans-species suppression. The indicated *S.cerevisiae* strains were transformed with either pYES2 alone, pYES-pak1 or expression vectors encoding Ste20p Ste5p or Ste11p. Following mating with a tester strain, diploids were selected on leu$^-$ medium containing galactose.

Materials and methods

Strains and media

Schizosaccharomyces pombe cells were grown in complex medium, YEA (Gutz *et al.*, 1974) or in Ediburgh minimal medium (EMM) lacking uracil, leucine or both (Sherman *et al.*, 1978). The haploid strains FWP165 and FWP172 (Table VI) were used to generate the diploid strain SOP1 used for gene disruption. The wild-type strain 972 (h^-) was used. *Saccharomyces cerevisiae* strains used in this work are listed in Table VI. *Saccharomyces cerevisiae* cells were grown on either minimal medium 0.67% (w/v) yeast nitrogen base supplemented with the appropriate amino acids or rich medium [1% (w/v) yeast extract, 2% (w/v) bactopeptone] containing 2% (w/v) of glucose or galactose.

PCR

The following degenerate oligonucleotides were used for PCRs: primer F, 5′-CGG-GAT-CCG-TNG-CNA-T(A/C/T)A-A(A/G)C-A(A/G)A-TGA-A-3′ (sense) and primer R, 5′-CGG-AAT-TCN-GGN-GG(C/T)-TCN-CC(C/T)-TC(A/G/T)-ATC-AT-3′ (antisense), which contain restriction sites for *Bam*HI and *Eco*RI, respectively, followed by codons for the amino acids VAIKQM and MIEGEPP, which are conserved in *S.cerevisiae* Ste20p and rat $p65^{pak}$. DNA amplification was performed in a Idaho Technology 1605 air thermo-cycler in a volume of 10 µl containing 50 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.1% BSA, 0.5 mM each primer, 0.4 U Vent polymerase (New England Biolabs) and 10 ng of DNA from an *S.pombe* cDNA library (Yamawaki-Kataoka *et al.*, 1989). Thirty cycles of amplification were performed (94°C for 1 s, 37°C for 1 s and 74°C for 30 s), and the resulting ~500 bp product was isolated from an agarose gel, reamplified under the same conditions, re-isolated, digested with *Bam*HI and *Eco*RI and subcloned into pUC19. The plasmids from 10 white colonies were isolated and the inserts sequenced. One plasmid contained an insert whose sequence closely resembled the catalytic domain of rat $p65^{pak}$ and *S.cerevisiae* Ste20p.

Table IV. $pak1^+$ suppresses the mating defect of a *ste20* null allele

Plasmid	Mating efficiency[a]		Percentage of STE20 (%)	
	A	B	A	B
pYES2	0.0	0	0.0	0
pFLC1 (STE20)	0.45 ± 0.05	0.25 ± 0.03	N/A[b]	N/A
pYES-pak1	0.13 ± 0.08	0.03 ± 0.01	28	12

[a]The results of two independent experiments are given (A and B). Four independent isolates were assayed in duplicate in each experiment.
[b]Not applicable.

Table V. Overexpression of inactive cdc42 or pak inhibit mating in *S.pombe*

Overexpressed alleles	Percentage mating (%)	
	+ Thiamine	– Thiamine
None	58.1 ± 2.2	56.3 ± 2.0
cdc42	54.7 ± 1.9	52.0 ± 1.9
$cdc42^{T17N}$	44.1 ± 1.5	4.5 ± 0.5
pak1	56.9 ± 2.4	55.3 ± 2.4
$pak1^{K415,416R}$	51.5 ± 2.4	3.8 ± 0.6

FY118 cells transformed with pREP constructs were grown for 5 days at 29°C on PM-N plates with appropriate auxotrophic supplements in the presence or absence of thiamine. The number of zygotes, asci and unmated cells were determined microscopically. The mating values represent averages from three independent determinations.

Isolation of cDNA clones

The ~500 bp product was labeled with [α-^{32}P]dCTP (New England Nuclear) to a specific activity of 2×10^8 c.p.m./µg by the random priming technique (Feinberg and Vogelstein, 1983). Twenty-five thousand colonies of a *S.pombe* cDNA library (Yamawaki-Kataoka *et al.*, 1989) were screened under high-stringency hybridization conditions by standard methods.

DNA sequence and peptide comparisons

The sequence of clone 112 ($pak1^+$) was determined by the dideoxy chain termination technique (Sanger *et al.*, 1977) using a combination of nested deletions (Henikoff, 1984) and oligonucleotide primers. The University of Wisconsin Genetics Computer Group (GCG) programs were used to compile and analyze the sequence data. Alignments were performed using the Gap, Pileup and Prettyplot comparison programs from the GCG program.

Southern and Northern blot analyses

Genomic *S.pombe* DNA was isolated using the protocol of Hoffman and Winston (1987). Five µg of DNA were digested with *Aat*II, electrophoresed and transferred to a nylon membrane and cross-linked using UV irradiation. For Northern blots, total RNA from haploid *S.pombe* cells was obtained using the protocol of Carlson and Botstein (1982). RNA (10 µg) was electrophoresed on a 1.0% agarose–6% formaldehyde gel, transferred to a nylon membrane and cross-linked using UV irradiation. Filters were hybridized overnight at 65°C in 0.5 M $NaPO_4$, pH 7.2, 7% SDS, 1% bovine serum albumin with full-length 3 kb [^{32}P]dCTP-labeled $pak1^+$ cDNA and washed twice with 0.2× SSC and 0.2% SDS at 65°C. Autoradiograms were obtained by exposing the blots to Kodak XAR film with intensifying screens at –70°C for 24 h.

Protein kinase assays

Wild-type *S.pombe* was transformed with the conditional expression vector pREP3X (Forsburg, 1993) encoding either wild-type or kinase-dead (K415R, K416R) HA epitope-tagged Pak1p. Site-directed mutations were created by the unique site elimination procedure (Deng and Nickoloff, 1992). The transformants were grown in leu$^-$ EMM (Moreno *et al.*, 1991) in the presence of 20 µM thiamine, then induced by growth in EMM minus thiamine to an mid-log phase. Protein extracts from 25 ml cells were obtained and 30 µg of protein from each sample were subjected to SDS–PAGE and transferred to a polyvinylidine fluoride (PVDF) membrane. Proteins bound to the membrane were renatured in 7 M guanidine–HCl and assayed for *in situ* protein kinase activity according to the method of Ferrell and Martin (1990). Phosphoamino acid analysis was performed on the excised protein band by standard methods (Boyle *et al.*, 1991).

Table VI. Yeast strains

Strain	Genotype	Source
Schizosaccharomyces pombe		
FWP165	*h^+ ura4-D18 leu1-32 ade6-M216*	C.Hoffman
FWP172	*h^- ura4-D18 leu1-32 ade6-M210*	C.Hoffman
FY118	*h^{90} ura4-D18 leu1-31 ade6-M216*	this study
Saccharomyces cerevisiae		
YEL-337-3B	*MATa ade2 his3 leu2 trp1 ura3 can1 ste20::TRP1*	E.Leberer
βSTE5Δ	*MATa ura3-52 lys2 leu2 trp1-289 his3Δ-200 met GAL$^+$ ste5::HIS3*	A.Levitzki
E929-6C-20	*MATa ste11-6 cyc1 CYC7-H2 can1 leu2-3,112 trp1Δ1 ura3-52*	B.Errede
RSY16	*MATα ade2-1 leu1-c lys2-1 trp5-20 ura1*	R.Strich

Immunoblots

Proteins were separated on 10% SDS–polyacrylamide gels, transferred to a PVDF membrane and blocked with 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20 (TBST) containing 5% non-fat dry milk. The membrane was then incubated in 1 μg/ml anti-HA (12CA5) or anti-myc (9E10) mAbs at room temperature for 1 h, washed three times with TBST, incubated in 1:10 000 dilution of horseradish peroxidase-conjugated goat anti-mouse antibody (AlphaQuest) for 45 min and again washed three times with TBST. The immunoblot was then developed using chemiluminescence according to the manufacturer's protocol (Amersham Inc.).

In vitro binding assay

pGEX-Cdc42 bacterial expression vectors were created by subcloning PCR amplification products of *S.pombe cdc42* (Miller and Johnson 1994) into *Bam*HI- and *Eco*RI-digested pGEX-2T (Smith and Johnson, 1988). pGEX-Pak bacterial expression vectors were created by subcloning the putative PBD (amino acids 137–227) into pGEX-2T. Production and purification of GST fusion proteins were accomplished by standard techniques (Smith and Johnson, 1988) except that, in the case of pGEX-Cdc42, 1 μM GDP was included in the lysis buffer. Glutathione–agarose-bound purified GST or GST–Cdc42p proteins were loaded with guanine nucleotides by incubating the beads in a solution containing 100 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 120 μM GDP or GTP-γS, 50 mM NaCl, 1 mM DTT, 5% glycerol and 0.1% Triton X-100. Protein extracts from induced *S.pombe* cultures were incubated with the loaded beads for 2 h at 4°C. The beads were then washed extensively with PBST plus 10 mM $MgCl_2$, then boiled in 1× SDS–PAGE buffer for 5 min. The samples were separated on a 10% SDS–polyacrylamide gel and processed for immunoblot as above using either anti-HA or anti-Myc mAbs.

Interaction trap binding assays

Full-length *pak1*$^+$ cDNA was subcloned into the bait vector pEG202 (Gyuris *et al.*, 1993). Wild-type and mutant forms of *S.pombe cdc42* cDNA were subcloned into the activation domain plasmid pJG4-5 (Gyuris *et al.*, 1993). Bait vectors, activation domain vectors and a *lacZ* reporter were co-transformed into EGY48 and transformants were selected on dextrose-containing medium lacking uracil, leucine and histidine. Four independent colonies from each transformation were analyzed for reporter activation. The colonies were replica-plated to galactose-containing medium to induce production of the bait protein and the colonies were assayed for β-galactosidase activity (Rose *et al.*, 1990) and for growth on medium lacking leucine.

Overexpression phenotypes of pak1 and cdc42 alleles

Various combinations of *pak1*$^+$, *pak1* ^{K415,416R}, *cdc42*$^+$, *ccdc42*^{T17N} and *cdc42*^{Q61L} (Table I) under the control of the thiamine-repressible *nmt1*$^+$ promoter were introduced into *S.pombe* FWP172 cells. The starting plasmids for the constructs were pREP3X (*LEU2*-based) and pREP4X (*ura4*$^+$-based).

To observe growth of cells expressing these combinations, transformants were successively subcultured three times to selective plates minus thiamine. To observe the overexpression phenotype, 5 ml cultures were grown in EMM + adenine in the presence of 15 μM thiamine to mid-log phase (OD ~1) and washed twice with 5 ml of water. The cells were then resuspended in 5 ml at OD 0.5 and grown to mid to late log (OD 1–2). These cultures were diluted to OD 0.2 and grown overnight twice. Final cultures were microscopically examined while in mid-log phase (OD 0.2).

Wet mounts of the cells were examined and photographed using an Olympus BH-2 microscope equipped with Hoffman Modulation Contrast optics. Two-hundred cells from each culture were assigned to morphological categories based on size and shape. 'Small round' cells were <6 μM in diameter. 'Large rounded' cells have a short axis >6 μM and included both spherical and ovoid cells. The 'irregular' category seen with *pak1*$^+$ overexpression deviated from the wild-type uniform rod morphology, having swellings, bends and/or strictures and tending to be longer than average wild type cells. Formaldehyde fixation and actin staining with rhodamine-conjugated phalloidin have been described (Alfa *et al.*, 1993).

Gene disruption

The plasmid pCG1 (Grimm *et al.*, 1988), containing the *S.pombe ura4*$^+$ gene, was digested with *Hin*dII, and the 1.8 kb *ura4*$^+$ fragment was blunt-ended and subcloned into *Nde*I- and *Bst*EII-cut, blunt-ended *pak1*$^+$. This manipulation replaces a large portion of the amino-terminus and kinase catalytic domain (amino acids 101–473) with *ura4*$^+$. Adiploid *S.pombe* strain was constructed and transformed with the *pak1*$^+$*::ura4*$^+$ fragment.

Saccharomyces cerevisiae mating assays

Saccharomyces cerevisiae strains bearing deletions in *STE20* or *STE5* or a mutation in *STE11* (Table VI) (Rhodes *et al.*, 1990; Leberer *et al.*, 1992; Perlman *et al.*, 1993) were transformed by a lithium acetate procedure (Gietz *et al.*, 1992) with a galactose-inducible expression vector, pYES2 (Invitrogen), bearing either no insert or cDNA encoding full-length Pak1p or a plasmid bearing the appropriate wild-type yeast gene [STE20-pFLC-1 (Ramer and Davis, 1993), STE5-p2-1PN (Perlman *et al.*, 1993) or STE11-pNC192 (Rhodes *et al.*, 1990)]. Transformants were selected for on minimal medium lacking uracil and complementation was assessed by mating with tester strain RSY16. Quantitative mating assays were performed essentially as described elsewhere (Sprague, 1991) except that mating was carried out on rich medium containing galactose as the carbon source and the mating time was increased to 7 h. Diploids were selected by growth on minimal medium lacking leucine. Patch mating was performed using standard techniques (Sprague, 1991).

Schizosaccharomyces pombe mating assays

The h^{90} strain FY118 (Table VI) was transformed with pREP vectors bearing either *pak1*$^+$, *pak1* ^{K415,416R}, *cdc42*$^+$ or *cdc42*^{T17N}. Transformants were grown in liquid PM medium (Gutz *et al.*, 1974) lacking leucine or uracil to mid-log phase, then plated on PM plates lacking nitrogen to induce sexual activity. After 5 days of growth at 29°C, the cells were examined microscopically and zygotes, asci and unmated cells were quantified.

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Note added

After submission of this article, Marcus *et al.* [(1995) *Proc. Natl Acad. Sci. USA*, **92**, 6180–6184] reported the cloning and characterization of a similar gene (*shk1*) from *S.pombe*. Their isolate encodes a protein identical to that reported here, except that it lacks the amino-terminal 118 amino acids.